Docket No.: 104831-0002-103
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Chang et al.

Confirmation No.: 9375

Application No.: 10/657383

Art Unit: 1623

Filed: September 8, 2003

Examiner: Maier, Leigh C.

For: METHOD FOR ENHANCING THE EFFECTIVENESS OF CANCER THERAPIES

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**Declaration Under 37 C.F.R. § 1.131 of Joseph Grimm**

Sir:

I, Joseph Grimm of hereby declare as follows:

1. I am the President of Prospect Therapeutics, Inc. ("Prospect"), which is the Assignee of the entire right, title and interest in the instant application. A Statement Under 37 C.F.R. § 3.73(b) establishing Prospect's ownership was submitted on May 22, 2007.

2. A Petition Under 37 C.F.R. § 1.47 was submitted to the Office on May 25, 2007 and a Request for Reconsideration, which included a declaration executed by me, was submitted on October 1, 2007. The Office granted the Petition Under 37 C.F.R. § 1.47 on November 7, 2007.

3. On information and belief, the inventors completed the invention as described and claimed in the above-identified application prior to March 27, 2001.

4. In support of this, I include herewith as Exhibit A a protocol design for a study, which I believe to have been carried out at the inventors' direction, designed to test the efficacy of interferon-α2b (IFN-α2b), GBC590B, and combinations thereof in a pancreatic carcinoma xenograft mouse model. IFN-α2b is an oncolytic cytokine, and GBC590B is a modified pectin that comprises a polymeric backbone having side chains terminated by galactose or arabinose units.

5. Exhibit **B** shows the results of this study. As can be seen, at the end of one week, the tumor size in all groups averaged 113-114 mg. However, as the experiment progressed, the average tumor size in groups receiving both GBC590B and interferon consistently lagged behind that of those receiving IFN or GBC-590 alone. By Day 18, the last date when all animals in these groups still survived, the mice receiving only IFN (Group 3) had tumors averaging 958.7 mg, while those receiving IFN with GBC-590 had tumors averaging 916.6 mg, 832.5 mg, and 906.9 mg, indicating that tumor growth was slower in these groups. At subsequent measurement times, after the death of some of the mice, the disparity increased dramatically, indicating that the combined therapy was particularly effective in slowing tumor growth in some of the mice. As then summarized in Exhibit **C**, administration of either therapy alone was insufficient to achieve a significant improvement in the lifespan (MDS, mean day of survival) of the test mice (i.e., the difference was within the margins of error), and no mice survived to the end of the experiment. In contrast, a combination of the therapies resulted in survival of some of the test mice, and in fact the combination allowed a lower dose of IFN-α2b to be used efficaciously. Indeed, two mice survived at lower doses of IFN-α2b (Groups 5 and 6) than at the dose that was, by itself, unable to achieve any significant benefit (Groups 3 and 4). Although the MDS does not show improvement, this number is calculated *excluding* the mice that survived (20% of the total test mice for groups 5 and 6). Accordingly, the results demonstrate that GBC590B enhances the efficacy of IFN-α2b, and in particular, enhances its ability to inhibit tumor growth.

6. By the time of the study described above, it was generally known in the art that modified pectin binds galectins, such as galectin-3, through its galactose residues and that other galectin-binding carbohydrates would be expected to have similar biological activities. For example, an article by Platt (an undersigned co-inventor of the instant application) and Raz ("Modulation of the Lung Colonization of B16-F1 Melanoma Cells by Citrus Pectin," Journal of the National Cancer Institute, 84: 438-442 (1992), Exhibit **D**) discusses a prior study showing that galactoside-binding lectins have been shown to mediate cell-cell adhesion and cell-extracellular matrix adhesion through carbohydrates containing terminal galactosyl residues. The article reports another prior study that liver metastasis of murine L-1 sarcoma cells was inhibited by D-galactose and arabinogalactan. Based upon this prior work, the article evaluates molecules rich in galactoside residues for modulating tumor cell colonization *in vivo*. In addition, U.S. Patent No. 5,834,442 (Exhibit **E**), filed July 7, 1994 and issued November 10, 1998, states that it had been previously demonstrated that modified citrus pectin could interfere with cell-cell interactions mediated by cell surface carbohydrate-binding galectin-3 molecules. This patent then teaches that complex carbohydrates rich in galactoside residues, such as pectin, act as potent inhibitors of prostate carcinoma metastasis. Furthermore, U.S. Patent No. 5,681,923 (Exhibit **F**), filed October 6, 1995 and issued October 28, 1997, for which co-inventor Platt is the sole inventor, discloses the sequence of galactose-specific binding polypeptides and the description of Figure 1 teaches that galactose bound to such polypeptides can be a simple sugar or a portion of a polysaccharide. Based on the inventors' knowledge of these facts and the results described in paragraphs 3 and 4, it is my belief that the inventors expected that galectin-binding carbohydrates generally, particularly those containing terminal galactose moieties, would be useful in the invention.

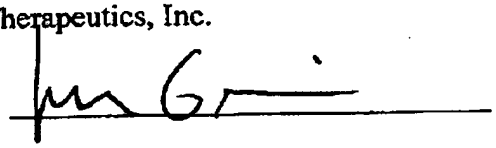
7. To the best of my knowledge, the results described in paragraph 4 were obtained in the United States through experiments performed by the inventors in collaboration with researchers

working under the inventors' direction, and were obtained in a report dated prior to March 27, 2001. The dates redacted from Exhibit **B** are all prior to March 27, 2001.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Joseph Grimm, President,
Prospect Therapeutics, Inc.

Dated: 5/27/08

Signature: 

## Table 1

Protocol Design for the Panc-e20 Study

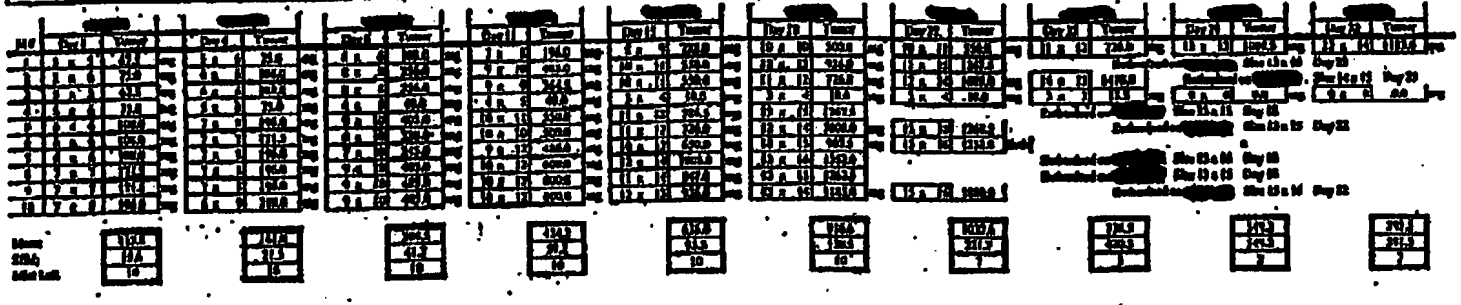
| Group | n | Treatment Regimen 1 | | | | Treatment Regimen 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Agent | mg/kg | Route | Schedule | Agent | mg/kg | Route | Schedule |
| 1 | 10 | Vehicle | — | iv | D1,2,4,6,8,10,12,14 | — | — | — | — |
| 2 | 10 | GBC590B | 6.4 | iv | D1,2,4,6,8,10,12,14 | — | — | — | — |
| 3 | 10 | IFN-α2b | $10 \times 10^6$ Units/kg | sc | qd x 14 | — | — | — | — |
| 4 | 10 | GBC590B | 6.4 | iv | D1,2,4,6,8,10,12,14 | IFN-α2b | $10 \times 10^6$ Units/kg | sc | qd x 14 |
| 5 | 10 | GBC590B | 6.4 | iv | D1,2,4,6,8,10,12,14 | IFN-α2b | $5 \times 10^6$ Units/kg | sc | qd x 14 |
| 6 | 10 | GBC590B | 6.4 | iv | D1,2,4,6,8,10,12,14 | IFN-α2b | $2.5 \times 10^6$ Units/kg | sc | qd x 14 |

Exhibit A

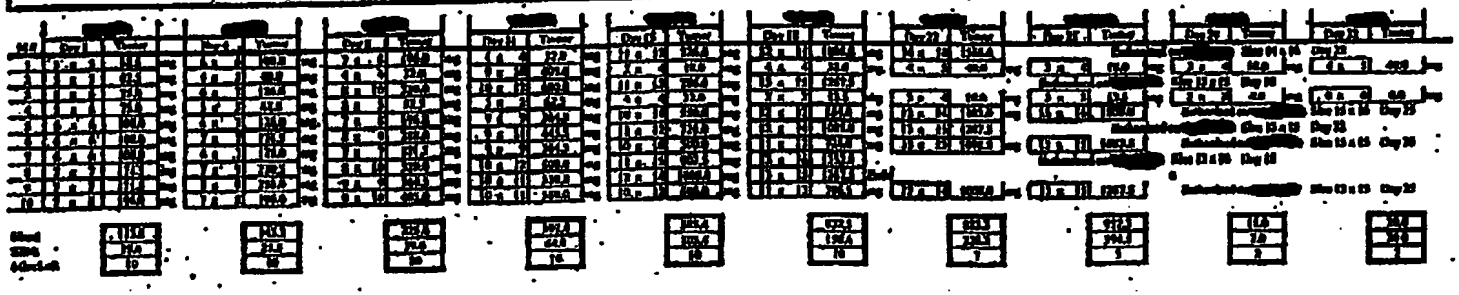
# Exhibit B

Experiment Number: Pano-c20; Technician(s): R. Ball; The Experiment Started on: [redacted]

Group 4: CDC590 (6.4 mg/kg) and IFN-α2b (10x10e6 U/kg mg/kg)



Group 5: CDC590 (6.4 mg/kg) and IFN-α2b (5x10e6 U/kg mg/kg)



Group 6: CDC590 (6.4 mg/kg) and IFN-α2b (2.5x10e6 U/kg mg/kg)

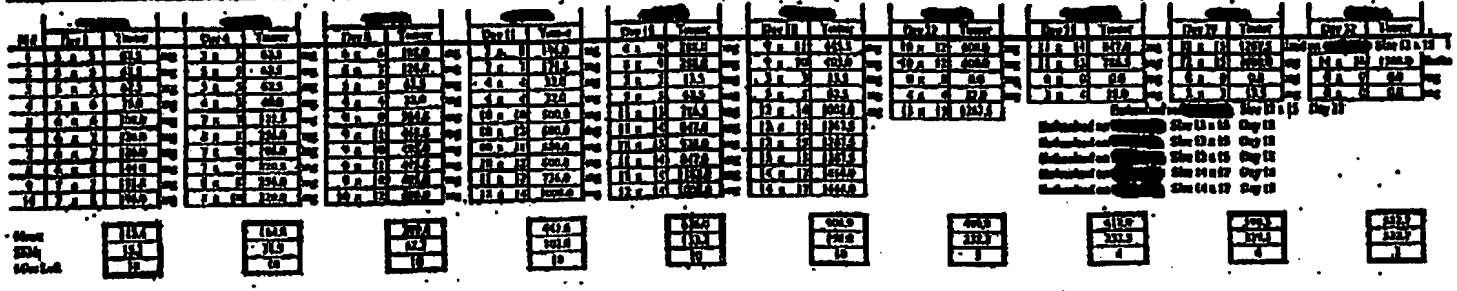


**Table 2**

Response Summary for the Panc-e20 Study

| Group | n | Regimen 1 | | Regimen 2 | | MDS to 1.2 g ± SEM (n) | # Toxic Deaths | # of Survivors | # CR | # PR | # Stable Disease |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Agent | mg/kg | Agent | mg/kg | | | | | | |
| 1 | 10 | Vehicle | --- | --- | --- | 22.6 ± 1.9 (9) | 1[a] | 0 | 0 | 0 | 0 |
| 2 | 10 | GBC590B | 6.4 | --- | --- | 23.0 ± 2.4 (10) | 0 | 0 | 0 | 0 | 0 |
| 3 | 10 | IFN-α2b | $10 \times 10^6$ Units/kg | --- | --- | 21.9 ± 1.8 (10) | 0 | 0 | 0 | 0 | 0 |
| 4 | 10 | GBC590B | 6.4 | IFN-α2b | $10 \times 10^6$ Units/kg | 20.9 ± 1.6 (9) | 0 | 1 | 1 | 0 | 0 |
| 5 | 10 | GBC590B | 6.4 | IFN-α2b | $5 \times 10^6$ Units/kg | 20.1 ± 1.0 (8) | 0 | 2 | 2 | 0 | 0 |
| 6 | 10 | GBC590B | 6.4 | IFN-α2b | $2.5 \times 10^6$ Units/kg | 20.3 ± 2.0 (8) | 0 | 2 | 2 | 0 | 0 |

[a]The mouse escaped and was euthanized.

Exhibit C

**Exhibit D**

Table 3. Toxic effects of MST-16 therapy

| Toxic effect | No. of patients (%) | Toxicity grade 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Leukopenia | 19 (70) | | 4 | 7 | 8 |
| Anemia | 14 (52) | 1 | 3 | 8 | 2 |
| Thrombocytopenia | 12 (44) | 2 | | 5 | 5 |
| Elevation of aspartate aminotransferase/ alanine aminotransferase | 4 (15) | 1 | 3 | | |
| Elevation of total bilirubin level | 1 (4) | | 1 | | |
| Nausea/vomiting | 10 (37) | 7 | 3 | | |
| Anorexia | 10 (37) | 2 | 6 | 2 | |
| Diarrhea | 9 (33) | 4 | 5 | | |
| Stomatitis | 5 (19) | 1 | 2 | 1 | 1 |
| Alopecia | 2 (7) | 1 | 1 | | |
| Pyrexia | 1 (4) | 1 | | | |

Furthermore, studies of combination chemotherapy with other antitumor drugs are warranted, since, in Japan, MST-16 has been shown to have antitumor activity in combination with other drugs in vitro and in vivo. These studies have demonstrated supra-additive effects on in vitro growth of MOLT-3 cells when the drug was used in combination with doxorubicin, amsacrine, and bleomycin, as well as additive effects with cyclophosphamide, cisplatin, mitomycin-C, and cytarabine (*19*). MST-16 has also had supra-additive effects on L1210 leukemia in mice in combination with doxorubicin, mitomycin-C, cisplatin, cyclophosphamide, and cytarabine (*20*). In addition, it is being used against breast cancer, gastric cancer, and adult T-cell leukemia/lymphoma in phase II trials in Japan.

## References


(1) CREMSTON AM, HELLMANN K, WHITEHOUSE S: Antitumour activity in a series of bis-dioxopiperazines. Nature 221:384–385, 1969

(2) HELLMANN K, NEWTON KA, WHITMORE DW, ET AL: Preliminary clinical assessment of ICRF 159 in acute leukaemia and lymphosarcoma. BMJ 1:822–824, 1969

(3) CRAVEN PJ, ALLEN LM, ALFORD DA: The bioavailability in man of ICRF-159, a new oral antineoplastic agent. J Pharm Pharmacol 27:914–918, 1975

(4) BELLER RE, EINSTEIN PF, CATALANO RH, ET AL: Phase II study of ICRF-159 in patients with metastatic colorectal carcinoma previously exposed to cytotoxic chemotherapy. Cancer Treat Rep 60:1395–1397, 1976

(5) O'CONNELL MJ, BEGG CB, SILVERSTEIN MN, ET AL: Randomized clinical trial comparing two-dose regimens of ICRF-159 in refractory malignant lymphomas. Cancer Treat Rep 64:1355–1358, 1980

(6) CAI JC, SHU HL, TANG CP, ET AL: Synthesis and antitumor properties of N1-acyloxymethyl derivatives of bis(2,6-dioxopiperazines). Chem Pharm Bull 37:2976–2983, 1989

(7) NARITA T, YAGUCHI S, KOMATSU T, ET AL: Antitumor activity of MST-16, a novel derivative of bis(2,6-dioxopiperazine), in murine tumor models. Cancer Chemother Pharmacol 26:193–197, 1990

(8) TSUKAGOSHI S, INABA M, TAKASE M, ET AL: Antitumor activity and mode of action of MST-16, a new antitumor agent. *In* Progress in Antimicrobial and Antitumor Chemotherapy (Proc 15th International Congress of Chemotherapy), vol 3 (Hirkarda S, Kammuale HP, eds). Federal Republic of Germany: Landsberg/Lech, 1987, pp 226–228

(9) TAKASE K, HIROAMI Y, ANDOH T: Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. Cancer Res 51:4903–4908, 1991

(10) ISHIDA R, MIKI T, NARITA T, ET AL: Inhibition of intracellular topoisomerase II by antitumor bis (2,6-dioxopiperazine) derivatives: Mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. Cancer Res 51:4909–4916, 1991

(11) FUKUE H, NIIYAMI H, NAKAO I, ET AL: Phase I study of MST-16. Jpn J Cancer Chemother 17:1287–1294, 1990

(12) MASAOKA T, FUKUE H: MST-16 for the treatment of lymphoma. Proc 15th Int Cancer Congr of UICC, Hamburg, part II, 1990, p 607

(13) WORLD HEALTH ORGANIZATION: WHO Handbook for Reporting Results of Cancer Treatment. WHO Offset Publication No. 48. Geneva: WHO, 1979

(14) The non-Hodgkin lymphoma pathologic classification project: National Cancer Institute-sponsored study of classification of non-Hodgkin lymphomas: Summary and description of a working formulation for clinical usage. Cancer 49:2112–2135, 1982

(15) NARITA T, KOIDE Y, YAGUCHI S, ET AL: Antitumor activities and schedule dependence of orally-administered MST-16, a novel derivative of bis(2,6-dioxopiperazine). Cancer Chemother Pharmacol. In press

(16) SHARPE HBA, FIELD EO, HELLMAN NK: Mode of action of the cytostatic agent "ICRF 159." Nature 226:524–526, 1970

(17) TRANSONOV F, DABZYNKIEWICZ Z, MELAMED MR: Effects of the L isomer (+)-1,2-bis(3,5-dioxopiperazine-1-yl)propane on cell survival and cell cycle progression of cultured mammalian cells. Cancer Res 41:4566–4576, 1981

(18) DEVITA VT JR, HUBBARD SM, LONGO DL: The chemotherapy of lymphomas: Looking back, moving forward—The Richard and Linda Rosenthal Foundation award lecture. Cancer Res 47:5810–5824, 1987

(19) KANO Y, NARITA T, SUZUKI K, ET AL: The effects of MST-16 in combination with other anticancer agents in vitro. Proc 17th Int Congr Chemother, Berlin, 1991, abstr 81

(20) NARITA T, YAGUCHI S, KIMURA S, ET AL: Relationship between administration schedule and antitumor activity of MST-16. Proc 48th Annu Meet Jpn Cancer Assoc, Tokyo, 1989, p 379


March 18, 1992

# Modulation of the Lung Colonization of B16-F1 Melanoma Cells by Citrus Pectin

*David Platt, Avraham Raz**

***Context:*** Studies have shown that the galactoside-containing simple sugars and anti-galactoside-binding lectin antibodies may affect experimental tumor cell metastasis. However, the limited number of reagents used thus far necessitate further observations. ***Purpose:*** Natural citrus pectin (CP) and pH-modified CP (MCP), rich in galactose residues, were used to study the involvement of carbohydrates containing galactoside residues in cellular interaction in vitro and in lung colonization in vivo of B16-F1 melanoma cells. ***Methods:*** B16-F1 melanoma cells were incubated with various concentrations of CP and MCP. Their ability to form homotypic aggregation in vitro and tumor lung colonization in vivo in 8-week-old female C57BL/6 mice was then analyzed. ***Results:*** The CP binds to the surface of B16-F1 melanoma cells; this binding can be inhibited by lactose at a concentration of 0.15 *M*. Intravenous injection of the murine B16-F1 melanoma cells with the natural CP resulted in a significant increase (up to threefold) in the appearance of tumor colonies in the lung and in increased homotypic aggregation properties of the cells, while injection of MCP significantly decreased B16-F1 experimental metastasis (>90%). ***Conclusions:*** Tumor galactoside-binding proteins mediate cellular recognition by linking oligosaccharides with terminal D-galactoside residues on adjacent cells. Successful interference

with such a process with M... may lead to a reduced ability to form tumor cell emboli and metastasis. *Implications:* These findings imply that the galactose-containing carbohydrate side chains of CP might mimic or compete with the natural ligand(s) of the tumor galactoside-binding protein (gal-lectin) and thus affect cellular interactions relevant for metastasis. [J Natl Cancer Inst 84:438–442, 1992]

Previously, galactoside-binding lectins were shown to mediate cell-cell adhesion and cell-extracellular matrix adhesion through carbohydrates containing terminal or penulated galactosyl residues. The role of galactose residues and their complementary receptors in this process was previously demonstrated, leading to the exploration of their possible use for the understanding of and intervention in tumor metastasis (*1,2*). Experimental liver metastasis of the murine L-1 sarcoma cells was inhibited by D-galactose and arabinogalactan (*3*), while methyl-α-D-lactoside and lacto-*N*-tetrose caused significant reduction in the metastatic deposition of B16 melanoma cells compared with the control (*4*). Treatment of B16 melanoma and UV-2237 fibrosarcoma cells in vitro with monoclonal antibody directed against tumor galactoside-binding protein (gal-lectin) before their injection into the tail veins of syngeneic mice resulted in a marked decrease in the development of tumor colonies in the lung (*5*). Furthermore, a correlation was established between the level of a human gal-lectin and the serum level of carcinoembryonic antigen and the stage of progression of colorectal carcinoma in human patients. This correlation suggests a role for gal-lectin in human colon cancer (*6*).

In this investigation, we have used natural citrus pectin (CP) and pH-modified CP (MCP), molecules which are rich in galactoside residues, to further evaluate the possible use of carbohydrate-containing galactosyl residues for augmenting tumor cell colonization in vivo.

## Materials and Methods

### CP and Its Modification

CP (70-100 kd; 0.5%; Sigma Chemical Co., St. Louis, Mo.; 10% methoxyl groups) was solubilized and sterilized under UV radiation for 48 hours. The total carbohydrate level was determined by the phenol sulfuric acid method (*7*). The pH of CP was modified by increasing the pH to 10.0 with NaOH (3 N) for 30 minutes and then by decreasing it to 3.0 with HCl (3 N) according to the method of Albersheim et al. (*8*). Samples were taken after 10 hours and 24 hours, and the pH of the samples was equilibrated to 6.3. The solutions were washed with ethanol (70%) and dried with acetone (100%), resulting in MCP fragments of approximately 10 kd. A sample of dried MCP was rehydrated with $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (pH 7.2) (CMF-PBS) to a final stock solution of 0.5% (wt/vol). The molecular weights of CP and MCP were determined by viscosity measurements (*9*) at 25 °C in an Ubbelohde No. 1 viscometer (Ubbelohde, The Netherlands) with sodium-hexametaphosphate at 20 m*M* (pH 4.5), 0.2% EDTA, and (0.9%) NaCl.

Neutral sugars in CP were estimated from the difference between the *m*-hydroxyphenol method (*10*) and the total carbohydrates with phenol sulfuric acid (*7*). The composition of the natural sugars was obtained by hydrolysis in trifluoroacetic acid (2 N). The respective alditol acetates were analyzed by gas-liquid chromatography as described (*8,11,12*). CP was radiolabeled by oxidation with $NaIO_4$, followed by reduction with $NaB^3H_4$ (*13*).

### Cells and Culture Conditions

B16-F1 melanoma cells (*13*) were grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, Inc., Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, and antibiotics. Cell cultures were incubated in a humidifier atmosphere of 7% $CO_2$ and 93% air. To ensure reproducibility, all experiments were performed with cultures grown for no longer than 6 weeks after recovery from frozen stocks.

### Lung Colonization Assay

B16-F1 cells grown to 70% confluence were detached with 2 m*M* EDTA in CMF-PBS. The cells were then washed and resuspended in CMF-PBS with or without CP and MCP, and aliquots of the suspension containing $1 \times 10^5$ cells in 0.2 mL were injected intravenously into the tail veins of 8-week-old female C57BL/6 mice. After 17 days, the mice were autopsied. The number of tumor colonies in the lung was determined under a dissecting microscope (*14*).

### Assay for CP-Induced Homotypic Aggregation

Cells were detached with 2 m*M* EDTA in CMF-PBS and suspended at $1 \times 10^5$ cell/mL in CMF-PBS as described (*7*) with and without 0.05% CP or 0.05% MCP. Aliquots containing 0.5 mL of cell suspension were placed in siliconized glass tubes and agitated at 50 rpm for 30 minutes at 37 °C. The aggregation was then terminated by fixing the cells with 1% formaldehyde in CMF-PBS. Samples were used for counting the number of single cells, and aggregation was calculated according to the following equation:

$$(1 - Nt/Nc) \times 100,$$

where *Nt* and *Nc* represent the number of single cells in the presence of the tested compounds and the number of single cells in the control buffer (CMF-PBS), respectively.

## Results and Discussion

The lodgment, attachment, and growth of blood-borne neoplastic cells depend largely on cell embolization. The arrest of intravenously inoculated aggregates of tumor cells leading to intense metastatic growth is much higher than that of single cells. Furthermore, several studies using the same B16-F1 melanoma cell system have demonstrated a correlation between the tendency of the cells to undergo inter-

Received August 21, 1991; revised December 6, 1991; accepted December 17, 1991.

Supported in part by grant CTR-2002 from the Council for Tobacco Research and in part by the Paul Zuckerman Support Foundation for Cancer Research to A. Raz.

Cancer Metastasis Program, Michigan Cancer Foundation, Detroit, Mich.

We think Ms. Nancy Squier for her critical evaluation of the manuscript and Ms. Vivian Powell for typing the manuscript.

*Correspondence to: Avraham Raz, Ph.D., Metastasis Research Program, Michigan Cancer Foundation, 110 E. Warren Ave., Detroit, MI 48201-1379.

cellular interactions in culture and their metastatic potential. Previously, we (*1*) suggested a molecular basis for such processes and demonstrated that several tumor cells, including the B16-F1 melanoma cells, contain galactoside-binding lectin which mediates cell homotypic aggregation in the presence of the asialoglycoproteins. Carbohydrates containing galactoside residues and antibodies directed against the gal-lectin were shown to reduce the tendency of tumor cells to develop metastases (*1-5*). The effect of CP on such processes was tested in the search for additional reagents for evaluation of the possible relationship between the gal-lectin and the endogenous ligand.

CP is a branched complex polysaccharide polymer responsible for the texture of fruits and vegetables. The CPs consist of partially esterified galacturonic acid residues with side chains composed of arabinose, galactose, glucose, mannose, and xylose. The sugar composition of CP would indicate that the anhydrogalacturonic acid comprises about 50% of the total residues, while galactose and arabinose constitute the two other major carbohydrates of CP, comprising 20% and 15%, respectively (Fig. 1). The modification of CP to MCP by pH involves degradation of the main galacturonic acid chain by β-elimination (high pH) followed by partial degradation of the natural carbohydrates (low pH), resulting in nonbranched carbohydrate chains of basically the same sugar composition of the unmodified CP (*8,15*).

The B16-F1 melanoma cells exhibited a low level of spontaneous homotypic aggregation, clearing a 1-hour agitation in CMF-PBS (Fig. 2, A). The aggregation of the cells, however, was markedly increased in the presence of 0.05% CP (Fig. 2, A). In contrast, an equal concentration of the nonbranched MCP failed to stimulate cell aggregation (Fig. 2, A). It is conceivable that the cell-surface gal-lectins recognize and bind galactosyl residues on different side chains of the same CP molecules, which serves as a cross-linking bridge between cells and subsequently leads to the formation of cell aggregates, while the nonbranched MCP fails to cross-link. The pectin used here is a structural cell wall polysaccharide present in all higher plants. It is primarily a polymer of D-galacturonic acid. The

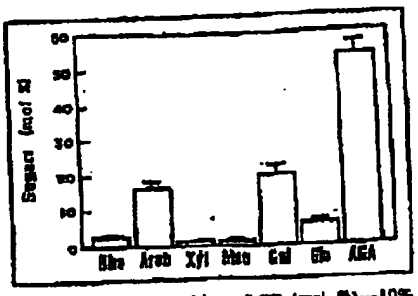



Fig. 1. Sugar composition of CP (mol %)—10% methoxyl group. The amount of galacturonic acid (AGA) was determined according to the method reported in (*10*), and total carbohydrate was ascertained by phenol sulfuric acid reaction according to the technique reported in (*7*). Total natural sugars were calculated from the difference between the two reactions based on galacturonic acid and glucose (Glu) standards. The composition and the amount of individual natural sugars were obtained by hydrolysis in trifluoroacetic acid (2 N). The respective alditol acetates were analyzed by gas chromatography according to the method reported in (*9*). Rha = rhamnose; Arab = arabinose; xyl = xylose; Man = mannose; Gal = galactose.

structural unit of all pectin molecules is a linear chain of (1-4)-linked α-D-galactopyranosyluronic acid (*8,13-16*). Further clarification of the nature of the interaction between the cells and CP came from studies that demonstrated a complete inhibition of [$^3$H]CP binding to cell surfaces in the presence of lactose (4-O-β-D-galactopyranosyl-D-glucose) (Fig. 2, B). Previously, it was shown that simple sugars, glycopeptides, and anti-lectin antibodies can inhibit the cell-cell aggregation (*5,17,18*).

We next tested the ability of the CP to affect the in vivo formation of B16-F1 tumor colonies in the lung. Cells were detached with 2 m*M* EDTA, suspended in CMF-PBS, and incubated on ice for 30 minutes with CMF-PBS, CP, and MCP. Aliquots of the suspension containing $10^5$ cells in 0.2 mL PBS were injected intravenously into the tail veins of syngeneic mice. After 17 days, the mice were autopsied, and the number of tumor colonies in the lung were counted (Table 1). A threefold increase in the number of tumor colonies in the lung was observed compared with the control experiment (CMF-PBS alone) when the B16-F1 cells were injected with CP (Table 1) and the effect of CP was dose dependent. To evaluate these findings further, the B16-F1 cells were exposed to and injected with MCP. Incubation of B16-F1 cells with 0.05% MCP resulted in a marked

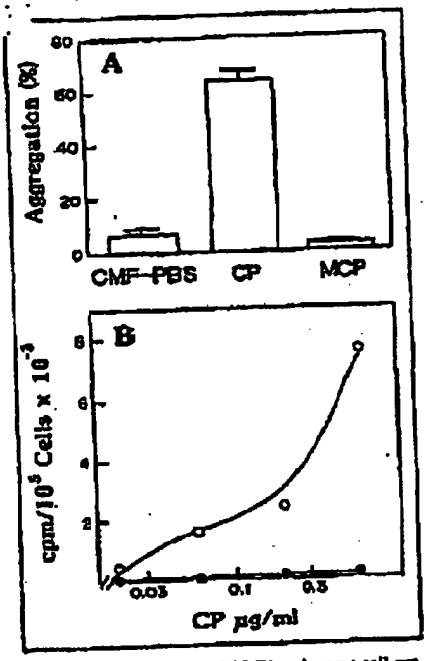



Fig. 2. Binding of CP to B16-F1 melanoma cell surface. A) CP-induced homotypic aggregation. Control CMF-PBS. CP—in the presence of unmodified CP (0.05%). MCP—in the presence of modified CP (0.05%). The cells were agitated for 60 minutes at 37 °C, and the degree of cell aggregation was determined as described in the "Materials and Methods" section. B) Binding of CP to B16-F1 cells: $10^5$ cells were incubated in the presence (●) or absence (○) of lactose (0.15 *M*) with different concentration of [$^3$H]CP (specific activity, $6.8 \times 10^6$ cpm/mg) for 30 minutes at 4 °C. The cells were washed three times in cold phosphate-buffered saline to remove unbound [$^3$H]CP. The cells were then solubilized with 0.1 N NaOH (30 minutes, 37 °C), and the radioactivity was determined in a β-counter. Each point represents the mean of triplicate experiments.

Table 1. Effect of CP and MCP on experimental lung metastasis of B16-F1 melanoma cells

| Treatment | No. of mice | Mean No. of lung tumor colonies per mouse (range) |
|---|---|---|
| | *Experiment 1* | |
| CMF-PBS | 12 | 43 (6-136) |
| CP, $5 \times 10^{-4}$%* | 12 | 74 (19-102) |
| CP, $5 \times 10^{-3}$%* | 10 | 80 (18-120) |
| CP, $5 \times 10^{-2}$%* | 10 | 112 (52-112)† |
| CP, $5 \times 10^{-1}$%* | 9 | 139 (68-172)† |
| | *Experiment 2* | |
| CMF-PBS | 43 | 33 (10-67) |
| MCP, $5 \times 10^{-2}$%* | 40 | 0 (0-1)† |
| MCP, $5 \times 10^{-1}$%* | 42 | 0 (0) † |

*Concentration in mol % (w/vol).

†$P < .01$ from the control (CMF-PBS) (two-tailed, Mann-Whitney *U* test).

decrease in the ability of these cells to form tumor lung colonization after their intravenous inoculation (Table 1). Fig. 3 shows that treatment with MCP led not only to a reduction in the absolute number of experimental metastases but also to an apparent reduction in the volume of the developed metastasis. The reason for the change in metastasis volumes observed following treatment with CP and MCP is not clear. It might result from faster or slower retention in the circulation, which may affect the onset of the growth of colonies. The inhibitory effect of MCP was not due to cell toxicity because no effect was observed in their in vitro growth properties when the cells were cultured with MCP or CP. Furthermore, injection of $10^5$ B16-F1 cells at a subcutaneous site in the presence or absence of MCP (0.5%) resulted in the same growth pattern of tumor formation, showing a cytotoxic effect of MCP in vivo (not shown).

Several studies using the same B16-F1 melanoma cell system have demonstrated a correlation between the tendency of cells to undergo intercellular interactions in culture and their metastatic potential [for review *see* (*1*)]. In vivo, intercellular adhesion by means of cell-surface lectin of one cell and carbohydrate-containing complementary molecules on an adjacent cell or by serum glycoproteins could serve as a bridge between adjacent cells and may contribute to tumor cell emboli-zation resulting, with increased organ colonization by the circulating tumor emboli.

The mammalian gal-lectin mediates the recognition process by linking to oligosaccharides with terminal-linked D-galactose residues (*19*). Investigators also found that somatic mutation, which blocks addition of gal and sialic acid to cellular glycoconjugates, as well as chemical inhibitors of *N*-linked processing, resulted in an impaired tumor cell adhesion to endothelial cells in vitro (*20*). Other investigators showed that the degree of GlcNAc α1–6Man α1-6Man α1-branching and the completion of these structures with SAβ2-3Gal β1-4 appear to be closely associated with metastatic ability (*20-23*) and that endothelial cells may have a lectin with similar specificity where the β1-4 Gal is part of a larger ligand structure (*23,24*). Those results indicate that β1-4 gal-lectin on microvascular endothelial cells can contribute to retention and secondary tumor formation of blood-borne tumor cells. In addition, galactosylation of D36W25 cells (*24*) increased the number of visible liver metastases after tumor cell injection by 30-fold. The unmodified CP may involve a recognition structure mechanism similar to the D36W25 cell-surface sugar.

The results presented here and in previous studies (*3,5*) are basically similar to experimental pyelonephritis, whereby infection with *Escherichia coli* can be inhibited by oligomannosides and mannan which bind to the mannose-specific lectins of *E. coli*, and binding of the bacteria to the uroepithelium is prevented (*25*).

We do not know whether CP and MCP compete with or resemble the yet unidentified natural ligand(s) of the mammalian gal-lectin; however, this study and those described earlier (*3-5*) may provide a new, simple modality for intervention with the successful colonization of circulating malignant cells.


Fig. 3. Experimental metastasis after intravenous injection of B16-F1 cells ($1 \times 10^5$) without CP (a) or with CP (b) or MCP (c). Eight-wk-old female C57BL/6 mice were given an intravenous injection of a 0.2-mL mixture of B16-F1 cells ($1 \times 10^5$) and sugar solution. The mice were killed 17 days after injection, and the tumor colonies per lung were measured under a dissecting microscope.

## References


(*1*) Raz A, Lotan R: Endogenous galactoside-binding lectin: A new class of functional tumor cell surface molecules related to metastasis. Cancer Metastasis Rev 6:433–452, 1987

(*2*) Gabius HJ: Discussion of functions of mammalian lectins—with emphasis on vertebrate lectins. Biochim Biophys Acta 1071:1–18, 1991

(*3*) Brown J, Ko HL, Ortiz K, et al: Inhibition of liver metastasis in mice by blocking hepatocyte lectins with arabinogalactan infusions and D-galactose. J Cancer Res Clin Oncol 113:51–55, 1987

(*4*) Ogata H, Toyokuni T, Dean B, et al: Effect of lactose derivatives on metastatic potential of B16 melanoma cells. Cancer Commun 2:311–316, 1990

(*5*) Meromsky L, Lotan R, Raz A: Implications of endogenous tumor cell surface lectins as mediators of cellular interactions and lung colonization. Cancer Res 46:5270–5275, 1986

(*6*) Irimura T, Matsushita Y, Sutton RC, et al: Increased content of an endogenous lactose-binding lectin in human colorectal carcinoma progressed to metastatic stages. Cancer Res 51:387–393, 1991

(*7*) Dubois MK, Gilles JK, Hamilton PA, et al: Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–361, 1956

(*8*) Albersheim P, Nevins JD, English PD, et al: A method for analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohydr Res 5:340–346, 1967

(9) CHRISTENSEN EP: Methods of grading p... in relation to the molecular weight (Intrinsic viscosity of pectin). Food Res 19:163–165, 1954
(10) BLUMENKRANTZ N, ASBOE-HANSEN G: New method for quantitative determination of uronic acids. Anal Biochem 54:484–489, 1973
(11) DRAVEL JD, MCNEIL M, ALBERSHEIM P: Structure of plant cell walls VIII. a new pectic polysaccharide. Plant Physiol 62:418–423, 1978
(12) DE VRIES JA, ROMBOUTS FM, VORAGEN AG, ET AL: Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. Carbohydr Polym 2:25–30, 1982
(13) GAHMBERG CG, HAYRY P, ANDERSSON LC: Characterization of surface glycoproteins of mouse lymphoid cells. J Cell Biol 68:642–653, 1976
(14) FIDLER IJ: Selection of successive tumour lines for metastasis. Nature New Biol 242:148–149, 1973
(15) ALBERSHEIM P, NEVINS H, DEUEL H: Splitting of pectin chain molecules in neutral solution. Arch Biochem Biophys 90:46–49, 1960
(16) BAUER HG, ASP NG, OSTE R, ET AL: Effect of dietary fiber on the induction of colorectal tumors and focal β-glucuronidase activity in the rat. Cancer Res 39:3752–3756, 1979
(17) ASAO MI, OPPENHEIMER SB: Inhibition of cell aggregation by specific carbohydrates. Exp Cell Res 120:101–110, 1979
(18) VICKER MG: BHK21 fibroblast aggregation inhibited by glycopeptides from the cell surface. J Cell Sci 21:161–173, 1976
(19) MERRLE, RK, CUMMINGS RD: Asparagine-linked oligosaccharides containing poly-N-acetyllactosamine chains are preferentially bound by immobilized calf heart agglutinin. J Biol Chem 263:16143–16149, 1988
(20) DENNIS JW, LAFERTE S, WAGHORNE C, ET AL: β 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science 236:582–585, 1987
(21) DENNIS JW, LAFERTE S, YAGEL S: Asparagine-linked oligosaccharides associated with metastatic cancer. Cancer Cells 1:87–92, 1989
(22) TAO TW, JOHNSON LK: Altered adhesiveness of tumor cell surface variants with reduced metastasizing capacity—reduced adhesiveness to vascular wall components in culture. Int J Cancer 30:763–766, 1982
(23) COHEN L, KERBEL RS, DENNIS JW: Tumor cell surface β 1-4-linked galactose binds to lectin(s) on microvascular endothelial cells and contributes to organ colonization. J Cell Biol 111:773–781, 1990
(24) ISHIKAWA M, DENNIS JW, MAN S, ET AL: Isolation and characterization of spontaneous wheat germ agglutinin-resistant human melanoma mutants displaying remarkably different metastatic profiles in nude mice. Cancer Res 48:665–670, 1988
(25) SHARON N: Lectin like bacterial adherence to animal cells. *In* Attachment of Organisms to the Gut Mucosa, vol I (Boedeker EC, ed). Boca Raton, Fla: CRC Press, 1984, pp 129–147

# Increasing Incidence of Primary Malignant Brain Tumors: Influence of Diagnostic Methods

*Marie Desmeules,* Tom Mikkelsen, Yang Mao*

*Background:* The incidence of brain cancer has increased dramatically over the last decades in most developed countries. Whether these trends can be attributed to improved diagnosis is not clear. *Purpose:* To determine the effect of new imaging technology on increased rates of brain cancer, we assessed the level of detection for neurological disorders when computed tomography (CT) and magnetic resonance imaging (MRI) results were not available. *Methods:* A neurologist performed a blind review of hospital charts from 356 randomly selected patients, hospitalized between 1985 and 1989 for neurological disorders, including brain cancer. All prediagnosis information except CT and MRI results was used as a basis for diagnostic re-evaluation. Also, a random sample of 151 brain cancer patients diagnosed between 1960 and 1965 was selected for a description of diagnostic methods used during that period. *Results:* A comparison between the original diagnoses and the re-evaluations for patients in the 1985-1989 sample indicated that there was, among the diseases selected, a 24% misclassification when CT scans and MRI were not available. In particular, 20% of brain tumors were undetected (95% confidence interval = 15%–25%), and 10% of non-tumor disorders were inaccurately labeled as brain tumors in the absence of these tests. The repeatability of the re-evaluations was 86%. *Conclusions:* Among elderly North Americans, at least twofold increases in brain cancer incidence were observed over the last two decades. Since our findings show that CT scans and MRI are responsible for the detection of about 20% of brain tumors, we conclude that other factors also are responsible for the observed trends. [J Natl Cancer Inst 84:442–445, 1992]

Brain cancer is often disabling and fatal. Rates of mortality from brain cancer have increased substantially, especially among the elderly (*1-4*), over the last decades in most developed countries. Whether such trends reflect a rise in brain cancer risk is controversial. Some investigators have attributed these increasing trends to improved diagnostic methods (*5-7*). Others argue that because of the magnitude of the increase and because brain cancer rates started to increase before the introduction of new imaging technology, the trends could not be due entirely to improved diagnostic methods (*1,2*).

Computed tomography (CT), introduced in the 1970s, may partly be responsible for increased tumor detection. Magnetic resonance imaging (MRI), which provides additional anatomic resolution, was introduced in the 1980s. MRI also can increase the rate of detection of tumors, in particular, those in regions of the brain such as the temporal lobe, the brain stem, and posterior fossa that are less easily visualized by other methods.

Numerous studies have assessed the diagnostic value of CT scans and MRI for intracranial disorders (*8-18*), mainly by comparing their accuracy with other methods of diagnosis. For example, it was found that CT scans had slightly higher sensitivity and specificity compared with radionuclide brain scans (*8*) and cerebral angiography (*9*) for the detection of brain tumors and cerebrovascular disease, in particular. The difference in these indices of accuracy between the two tests was only about 3%, however. Other studies indicated that the use of CT scans decreased the perceived need for

Received July, 19, 1991; revised November 14, 1991; accepted December 23, 1991.

M. Desmeules, Y. Mao, Bureau of Chronic Disease Epidemiology, Laboratory Center for Disease Control, Health and Welfare, Ottawa, Ont., Canada.
T. Mikkelsen, Ludwig Institute for Cancer Research, San Diego, Calif.
*Correspondence to: Marie Desmeules, M.Sc., Laboratory Center for Disease Control, Tunney's Pasture, LCDC Bldg., Rm. 22C, Ottawa, ON, K1A 0L2, Canada.